Alteration of the Growth of Cytomegalovirus and Herpes Simplex Virus Type 1 by Epidermal Growth Factor, a Contaminant of Crude Human Chorionic Gonadotropin Preparations

G. ERIC KNOX, DAVID W. REYNOLDS, STANLEY COHEN, and CHARLES A. ALFORD,
Departments of Obstetrics, Pediatrics, and Microbiology, University of Alabama in Birmingham Medical Center, Birmingham, Alabama 35294, and the Department of Biochemistry, Medical School, Vanderbilt University, Nashville, Tennessee 37203

ABSTRACT Pretreatment (12–48 h) of human fibroblasts with crude, human chorionic gonadotropin (HCG) was found to suppress cytomegalovirus infection and enhance productive herpes simplex type 1 (HSV) infection in vitro. Maximal effect on virus replication occurred at the time of maximal infectivity of control cultures (48 h and 6 days after viral innoculation for HSV and cytomegalovirus, respectively). The alteration in viral growth was not due to the HCG itself, but rather to epidermal growth factor, a contaminant of crude HCG. The effect of epidermal growth factor on viral infectivity was shown to be a cell-mediated event requiring protein synthesis.

INTRODUCTION

According to three large, epidemiological studies (1–3), genital excretion of cytomegaloviruses (CMV)[1] in young pregnant females increases significantly with advancing gestation. In our investigation, cervical shedding of CMV occurred in 1.5% of gravid women during the first trimester and then rose progressively throughout pregnancy until 13.5% were excreting virus near term (1). This increase in productive infection was hypothesized to be a reactivation of latent virus in the genital tract provoked by hormonal or other gestational factors. The hypothesis was based on the belief that CMV genital infection occurred in only 1.0% of young, nonpregnant females, a baseline incidence similar to that seen in early gestation (4). However, the documented frequency of genital shedding of CMV in our nonpregnant control population proved to be 10% (1). This frequency approximates that found in late gestation, and is significantly higher than the infection rate detected during the first trimester of pregnancy. Rather than progressive reactivation, suppression of genital CMV infection in early gestation, which diminishes as pregnancy progresses, is an alternative explanation for the observed epidemiologic findings (2, 3). Because hormonal changes remain a most attractive explanation for this suggested suppressive phenomenon, studies were undertaken to determine the effects of various pregnancy hormones on the growth of CMV in vitro. The initial investigations reported here employed commercially purchased, crude, human chorionic gonadotropin (HCG), a lyophilized preparation of human-pregnancy urine (5).

Crude HCG was chosen for three reasons. (*a*) HCG is the major gestational hormone present when CMV excretion is minimal (6). (*b*) The effect of HCG on viral replication has not been previously reported. (*c*) Crude HCG is composed of a complex mixture of biologically active substances other than HCG and, therefore, seemed to provide a good starting material to search for naturally occurring substance(s) which might suppress CMV replication. Indeed, others had already demonstrated depression of lymphocyte responsiveness to phytohemagglutinin stimulation (7) and alteration of

Received for publication 10 November 1977 and in revised form 30 January 1978.

[1] *Abbreviations used in this paper*: ara-C, cytosine arabinoside; BSC-1, green monkey kidney; CMV, cytomegalovirus; EGF, epidermal growth factor; HCG, human chorionic gonadotropin; h-EGF, human epidermal growth factor; HSV-1, herpes simplex virus type 1; LH, luteinizing hormone; m-EGF, mouse epidermal growth factor; MOI, multiplicity of input; PBS, phosphate-buffered saline; PFU, plaque forming units; poly(I):poly(C), polyriboinosinic-polyribocytidylic; VSV, vesicular stomatitis virus.

complement system activity in vitro (8) by contaminants of crude HCG.

Results presented here establish the fact that crude HCG can stimulate the growth of human fibroblasts in tissue culture, while concomitantly suppressing the replication of CMV and enhancing the production of herpes simplex virus type I (HSV-I). The active principle is not HCG, itself, but rather epidermal growth factor(EGF), a small peptide hormone excreted in human urine and apparently coisolated during the preparation of crude HCG.

METHODS

Cells. Discontinuous monolayer cultures of fibroblast cells, prepared in this laboratory from pooled human foreskins, and a continuous cell line of green monkey kidney (BSC-1), initially obtained from The American Type Culture Collection, were used throughout these experiments. Fibroblast cells were employed between passages eight and 13, and BSC-1 cells between passages 56 and 67. The cells were grown in sealed, plastic flasks (75 cm^2, Corning Medical, Corning Glass Works, Medfield, Mass.) at 37°C in an ambient atmosphere before experimental manipulations. Eagle's Minimal Essential Medium prepared with Hanks' salts and supplemented with 10% calf serum (inactivated at 56°C for 1 h), glutamine (2 mm/ml), and gentamicin (50 μg/ml) served as growth medium. When cells became confluent, they were maintained in Medium 199 with Earle's salts supplemented with 5% inactivated calf serum and glutamine and gentamicin in concentrations as noted above.

Viruses. The AD-169 strain of CMV and Indiana strain of vesicular stomatitis virus (VSV) (kindly supplied by Dr. Thomas Weller, Harvard University, School of Public Health) and a recent clinical isolate of HSV-1 (kindly typed by Dr. Andre Nahmias, Emory University) were employed for these investigations. All viral strains had been laboratory adapted in human fibroblasts before use.

Reagents. Crude HCG preparations (concentrations ~3,000 IU/mg) were purchased from Sigma Chemical Co., St. Louis Mo., Ayerst Laboratories, New York, Organon, Inc., West Orange, N. J., and Parke, Davis & Co., Detroit, Mich. Immunochemically pure lutenizing hormone (LH), HCG, and the Beta subunit of HCG were generously supplied by The National Pituitary Agency, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health. 10 lyophilized fractions of crude HCG (batch CR lot 121) prepared by gel filtration on a G-100 Sephadex column (Pharmacia Fine Chemicals, Piscataway, N. J.) were kindly supplied by Dr. Robert Canfield, Columbia University. According to an established biologic assay for HCG performed by Dr. Canfield (9), fractions 1–3 contained concentrated HCG (10,000 IU/mg), fraction 4 contained a much lesser amount (1,000 IU/mg), and fractions 5–10 had no measurable hormonal activity. These fractions as well as the other purified hormonal products were reconstituted in phosphate-buffered saline (PBS, pH 7.4) to contain 1 mg of dry wt material per ml and stored in small aliquots of −20°C before use. Purified mouse EGF (m-EGF), and specific rabbit antibody against m-EGF and human EGF (h-EGF) were prepared as previously described (10–12). Purified human fibroblast interferon and double-stranded polyriboinosinic-polyribocytidylic acid (poly [I]:poly [C]) were generously provided by Dr. George Galasso, Antiviral Substances Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Cycloheximide was purchased from Sigma Chemical Co. and cytosine arabinoside (ara-C) from the Upjohn Company, Kalamazoo, Mich. Except where indicated, reagents were diluted to specified amounts in maintenance medium just before use. Commercially supplied diluents were never employed to avoid possible adverse effects on cell and(or) viral growth due to the presence of various preservatives.

Hormone and drug treatment schedules. Cells were treated with specified amounts of crude HCG preparations and purified m-EGF at designated intervals, varying from 48 h before to 48 h after viral infection. With immunologically purified HCG, the Beta subunit of HCG, LH, and the Sephadex gel filtration separated fractions of crude HCG, 24 h pretreatment schedules were employed. All of these preparations were removed during the period of viral adsorption (90 min after inoculation) and then replaced and maintained in the fluid or agar overlay throughout the viral growth phase. Cells were pretreated 24 h with designated quantities of cycloheximide and ara-C with and without crude HCG or m-EGF. All of the latter drugs and hormones were removed before viral infection and not replaced thereafter. Crude HCG and m-EGF-treated controls were handled in the same manner in these latter experiments. With purified interferon, a 24-h pretreatment schedule was employed. Cells were treated with poly (I):poly (C) for 2 h followed 20 h after removal by virus challenge. Neither interferon or poly (I):poly (C) were added to the cultures after infection.

Growth of viruses and infectivity assays. Replication of infectious CMV, HSV-1, and VSV was assessed in human fibroblasts (2×10^5 cells) grown in wells of disposable plastic trays (Linbro Chemical Co., Hamden, Conn.) in a humidified 5% CO_2 atmosphere. Designated quantities of virus were inoculated in 0.05 ml amounts, allowed to adsorb for 90 min and, afterward, harvested in duplicate at specified intervals. Appropriate controls, as indicated for each experiment, were always included. Infectivity of clarified supernates collected from infected cells was used as a measure of extracellular virus. To quantitate total virus production, infected cells were scraped into their own media, combined, sonicated for 30 s at 25 W (model W185 Ultrasonic Instruments International Ltd., Farmingdale, N. Y.) and then the final clarified (2,000 rpm for 10 min at 4°C) product assayed for infectivity.

A modification of a previously described plaque-production assay technique was employed to quantitate viral infectivity (13). To measure CMV infectivity, fibroblast cells were utilized while BSC-1 cells were employed for HSV-1 and VSV infectivity. All cells were grown as described above. The overlay consisted of 1.5 ml of complete maintenance medium containing 2% methyl cellulose and was applied only once. Cells were fixed with 10% formaldehyde and stained with 0.03% methylene blue before counting at the following intervals after infection: day 8 for CMV, 48 h for HSV-1, and 24 h for VSV. Infectivity was expressed as plaque forming units (PFU) per ml.

The effect of purified HCG, LH, and the Beta subunit of HCG on CMV infectivity was assayed by a plaque-reduction technique (13). Plaque production was measured as described above, but a constant input (~100 PFU/0.05 ml) of virus was employed to infect untreated control cells and hormone-treated cells. Plaque production was compared in control and treated cells 8 days after infection, and differences were expressed as the percent plaque reduction.

Neutralization experiments. The following method was employed to determine if HCG or m-EGF caused extra-

cellular neutralization or inactivation of CMV. Equal parts of 0.2 ml of reconstituted crude HCG (50 IU/ml) or m-EGF (50 or 10 ng/ml), and virus pools (1,000 PFU/ml) were mixed and incubated for 1 h in 37°C water bath. The same quantities of virus pool and a 1:10 dilution of serum known to contain a high level of CMV neutralizing antibody, as well as virus plus diluent, served as positive and negative controls, respectively. Residual infectivities of the mixtures were assayed by the plaque-reduction technique previously described, except that end points were compared 2 wk after infection.

Immunofluorescence. Fibroblast monolayers grown to confluency on plastic tissue-culture slides (Lab-Tek Products, Miles Laboratories, Inc., Naperville, Ill.) were pretreated for 24 h with HCG (50 IU/ml), m-EGF (10 ng/ml), interferon (50 IU/ml), or maintenance medium and then inoculated with AD-169 strain of CMV at a multiplicity of input (MOI) of 0.01. After 1 h adsorption, the inoculum was aspirated and replaced with maintenance medium. At selected intervals, the slides were washed, fixed in acetone for 10 min at room temperature, then stained immediately or stored at −20°C for processing the following day. Monolayers were incubated with a 1:15 dilution of three CMV neutralizing antibody-positive (neutralizing titer range, 512–1,024) and three antibody-negative human sera for 1 h at 37°C. After thorough washing, slides were treated with a 1:30 dilution of fluorescein-conjugated, goat-anti-human IgG (F/P ratio, 5.0, prepared locally) for an additional hour, washed thoroughly, and mounted under a PBS-glycerol mixture. The specificity of the reaction observed in the absence of pretreatment with m-EGF, HCG, or interferon was established by the absence of fluorescence with three CMV negative sera, in sham-infected tissue, and in monolayers infected with heat-inactivated and U-V-irradiated virus. Positive sera demonstrated nuclear fluorescence in infected cells beginning 2 h postinfection (14).

Physicochemical characterization of the active principle in crude HCG. Because Sephadex G-100 prepared fraction no. 8 contained peak levels of the active compound under study but no demonstrable HCG activity, it was used for all physicochemical assessments. Lyophilized aliquots of fraction no. 8 were reconstituted to a concentration of 1 mg/ml and then subjected in 5-ml vol to dialysis and in 1-ml vol each to heat and trypsin treatments. Equal volumes of PBS were utilized to control for toxicity in each case. Dialyses were performed in Spectrapor membranes (mol wt cutoff 3,500 Spectrum Medical Industries, Inc., Los Angeles, Calif.) over a 48-h interval against three changes (1,000 ml each) of PBS. After dialysis, 1 ml of the dialysate was diluted 100:1 with PBS yielding a presumed concentration of 0.01 mg/ml of the starting product. This concentration had been previously shown to be maximally active in the viral assays employed. 1 ml of the reconstituted fraction was boiled for 10 min or mixed with 1 ml of 0.25% trypsin, pH 7.6, and allowed to incubate overnight at 37°C. Control PBS was treated in the same manner and untreated aliquots of fraction no. 8 were included in each experiment. Before examination the treated samples and controls were diluted and filtered.

Quantitation of cellular growth. After the various hormonal treatments, fibroblasts, grown as described previously in Linbro plates (Linbro Chemical Co.), were removed from wells at designated intervals with EDTA-trypsin (0.2 ml of a 0.25% solution) treatment for 5 min and the cell mixture gently pipetted to eliminate clumping. Medium 199 with 25%-inactivated, newborn-calf serum was then added. The cells were immediately pelleted by gentle centrifugation, washed, and resuspended in 1 ml of complete maintenance medium containing one part to three of trypan blue. Afterward, they were counted in a hemocytometer chamber. Each determination represented the average of the number of cells contained in three wells. Untreated cells served as controls, and exclusion of trypan blue was used to determine viability.

Fibroblast receptor assay for EGF. The assay is based on the ability of both mouse and human EGF to compete with ^{125}I-labeled m-EGF for binding sites on human foreskin fibroblasts as previously described in detail (11–12). Briefly, the assays were carried out on monolayer cultures of human foreskin fibroblasts (0.8 to 1.2 × 10^6 cells per 60 mm Falcon dish, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) by incubating a standard amount of ^{125}I-labeled m-EGF in the presence of aliquots of competing peptide or HCG preparations for 1 h at 37°C. Unbound ^{125}I-labeled m-EGF was removed by washing, the cells were solubilized by the addition of 1 ml of 0.5 M NaOH, and the radioactivity was measured with a gamma spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.). As previously reported (12), no competition with ^{125}I-labeled m-EGF could be detected with a wide variety of known peptide hormones, including HCG.

RESULTS

Studies with HCG preparations. The effect of 24 h-pretreatment of human fibroblasts with crude HCG on the replication of infectious CMV is depicted in Fig. 1. A dose-dependent reduction of total CMV infectivity was observed with increasing concentrations of crude HCG varying from 12.5 to 50 IU/ml. Though not shown, production of infectious extracellular virus was suppressed in like manner. Maximal reduction in infectivity (~2 logs) occurred at the highest concentration, 50 IU/ml of crude HCG, 6 days after infection. The degree of suppression was independent of viral input between multiplicities (MOI) of 0.01 to 0.5. Increasing the MOI to 1 reduced, but did not eliminate, the suppressive effect (1 log at 50 IU/ml HCG). Reduction in replication was greatest when the cells were pretreated with HCG for 24 h before viral infection. Pretreatment for less than 12 h or greater than 36 h reduced the amount of suppression, and treatment with or after infection did not influence growth of infectious virus. Data in the lower panel of Fig. 1 indicate that suppression of infectivity was not a result of cellular toxicity. Instead, crude HCG proved to be mitogenic for fibroblasts causing a 64% increase in cell number (2.00 × 10^5 control vs. 3.04 × 10^5 treated) with 98% of the cells remaining viable over the observation period. Preparations of HCG from Sigma Chemical Co., Ayerst Laboratories, and Organnon, Inc. gave similar results; the Parke, Davis & Co. preparation was toxic for fibroblasts.

To determine if HCG itself was the suppressive and(or) the mitogenic factor, CMV infectivity and cell growth were assessed after pretreatment of fibroblasts with Sephadex (Pharmacia Fine Chemicals)-purified HCG, immunochemically pure HCG, LH,

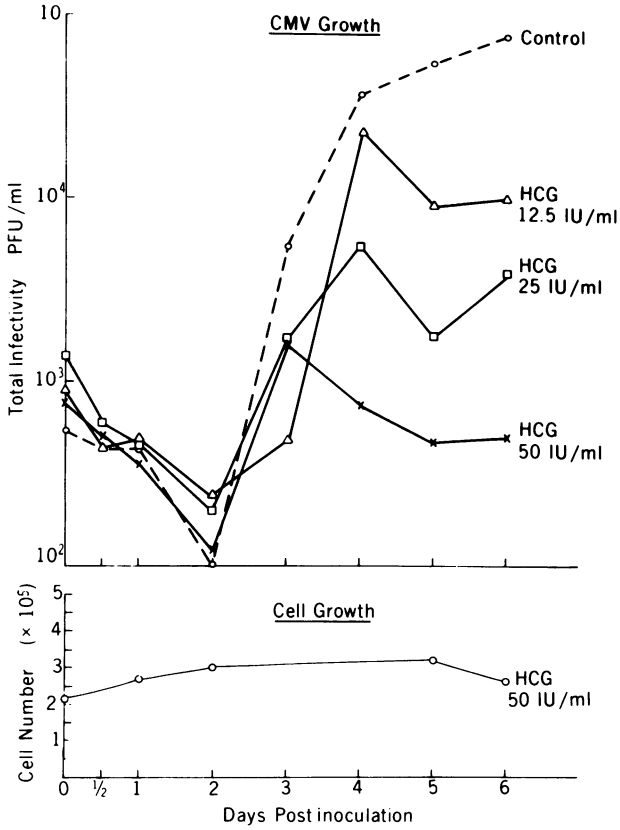


FIGURE 1 Effect of varying doses of crude HCG on growth of infectious CMV and human fibroblast cells. Experiments were performed when cells reached confluency, 5–7 days after subculture. Initial cell number was 2×10^5/well with a viral inoculum of 0.1 PFU/cell. Total CMV infectivity is depicted, but extracellular infectivity was suppressed in a like manner. Infectivity measurement and assessment of cellular number and viability are described under methods. Cell number in the untreated wells remained constant (2×10^5/well) throughout the experimental period.

and the Beta subunit of HCG. The results of these studies are shown in Table I. A 74% reduction of CMV plaque production was seen with crude HCG, whereas the purified HCG preparations, LH, or the Beta subunit of HCG had no effect on viral growth. In addition, none of the preparations, except crude HCG, were mitogenic for fibroblasts. Clearly, a contaminant(s) was the active material(s) under study. Assessment of Sephadex fractions showed that the viral suppressive and mitogenic factors were both isolated in two broad peaks with maximum activities found in fraction no. 4, containing low levels of HCG (1,000 IU HCG/mg) and fraction no. 8 containing no detectable HCG. Fraction no. 8 had maximal activities at a concentration of 0.01 mg dry wt/ml.

Suppression of CMV infectivity was not caused by antibody or other extracellular inactivating substances contaminating crude HCG, because replication was

TABLE I

Effect of Various Preparations of HCG and LH on the Growth of CMV in Human Fibroblasts

Treatment	Infectivity	Reduction
	PFU/0.05 ml	%
None, control	120	—
Crude HCG, 50 IU/ml	31	74
Purified HCG, 50 IU/ml		
gel filtration, fractions 1–3*	120	0
immunochemically pure‡	118	2
B subunit HCG‡	120	0
LH‡	122	0

* Sephadex G-100-purified crude HCG (fraction 1–3 containing 10,000 IU/mg).

‡ Immunochemically pure material obtained from the National Institute of Arthritis, Metabolism, and Digestive Diseases. The amount of LH and the B subunit of HCG was equivalent on a weight basis to 50 IU/ml of immunochemically purified HCG.

unaffected by 10 and 50 IU/ml of crude HCG in neutralization experiments. Neither the presence of interferon in the crude preparations nor interferon induction could account for the suppression, as evidenced by the data summarized in Table II. Extracellular production of VSV, the primary monitor for interferon, and HSV-1 were not suppressed after crude HCG treatment of fibroblasts. Instead, crude HCG consistently increased the extracellular production of HSV-1 and, to a much lesser extent, that of VSV. For example, in the experiment depicted in Table II, extracellular production of HSV-1 and VSV were increased by factors of 27 and three, respectively. As expected, CMV infectivity as measured by plaque production was reduced by 70%. In contrast to crude HCG, purified interferon and poly(I):poly(C) markedly reduced replication of all three viruses at concentrations of 50 IU/ml and 100 μg/ml, respectively. In fact, dose response studies indicated that as little as 1 IU/ml of interferon and 5 μg/ml of poly(I):poly(C) suppressed both CMV and VSV extracellular infectivity approximately 50%.

The factor that caused enhancement of HSV-1 replication was found to co-elute after gel filtration of crude HCG on G-100 Sephadex with the CMV suppressive and fibroblast mitogenic activities. Maximal activities of all were found in fraction no. 8. This fraction was therefore employed to better characterize the active material(s). From the results summarized in Table III, the HSV-1 enhancing property, the fibroblast mitogen, and the CMV suppressive factor all had molecular weights in excess of 3,500, were inactivated by trypsin, and resisted boiling, suggesting that these activities were mediated by the same or very

TABLE II

Comparative Effects of Crude HCG, Purified Fibroblast Interferon, and Poly (I):Poly (C) on Growth of VSV, HSV-1, and CMV in Human Fibroblasts*

Treatment	VSV		HSV-1		CMV	
	Infectivity	Reduction	Infectivity	Reduction	Infectivity	Reduction
	PFU/ml	%	*PFU/ml*	%	*PFU/0.05 ml*	%
None, control	2.5×10^7	—	3.5×10^4	—	200	—
HCG, 50 IU/ml	8×10^7	0‡	9.5×10^5	0§	60	70
Interferon, 50 IU/ml	0	100	8.8×10^3	85	2	99
Poly (I):Poly (C), 100 μg/ml	5×10^3	99	ND		4	98

* Fibroblasts were pretreated with HCG, interferon, or poly (I):poly (C), as described in Methods. Extracellular infectivity of VSV and HSV was determined 24 and 48 h postinfection, respectively, by titration onto BSC-1 monolayers. CMV infectivity was measured by plaque production as in Methods.

‡ Increased threefold.

§ Increased 27-fold.

similar proteins or polypeptides. All of the findings taken together suggested that the active contaminating material in crude HCG might be EGF, a small peptide hormone found in human urine which was known to be mitogenic for human fibroblasts in culture. Therefore, to determine if EGF was present in various HCG preparations, a sensitive radioreceptor assay for EGF was employed (11, 12). 50 IU of crude HCG contained 10 ng of EGF, and 0.01 mg of Sephadex fraction no. 8 contained 20 ng of EGF. The purified HCG preparations had no measurable EGF. The presence of EGF in crude HCG, in quantities known to be mitogenic for fibroblasts, further suggested that it was the active material under study and led to investigations to define the effects of purified EGF preparations on CMV and HSV-1 growth. Because of its ready availability and previously described biological similarity to h-EGF (10, 12), m-EGF was employed in these experiments.

Studies with EGF preparations. The effect of 24-h pretreatment of human fibroblasts with various concentrations of m-EGF on the replication of CMV and HSV-1 is depicted in Fig. 2. Productive infection was retarded with CMV and increased with HSV-1 in a dose-dependent fashion similar to that observed with crude HCG (Fig. 1). As little as 0.1 ng/ml of m-EGF was active, with maximal effects occurring with 10 ng/ml and remaining relatively constant up to 50 ng/ml. Total and extracellular infectivity with both viruses were similarly altered and independent of viral input between MOI of 0.01 to 1.0 for HSV-1, but the effect of MOI on CMV suppression was identical to that previously described for crude HCG. Maximal suppression of CMV growth occurred 6 days after infection and in 30 similar experiments varied from 1.04 to 2.84 (average 2.0±0.49) logs of extracellular infectious virus. As depicted in the data shown in Fig. 3, maximal enhancement of HSV-1 replication occurred at the time

TABLE III

*The Effect of Various Treatments on the Mitogenic, CMV Suppressive, and HSV-1 Enhancing Activities of Fractionated Crude HCG**

Preparation	Mitogenic activity		CMV infectivity‡		HSV-1 infectivity‡	
	Cell number	Increase	Decrease		Increase	
	$\times 10^5$	%	*PFU/ml* (log_{10})	%	*PFU/ml* (log_{10})	%
Control	2.00	—	5.69	—	5.76	—
Fraction no. 8*						
Untreated	3.00	50	3.07	>99	6.59	678
Dialysate	3.04	52	3.14	>99	6.70	879
Boiled (10 min)	3.08	54	3.30	>99	6.68	827
Trypsin (0.25%)	1.96	−2	5.69	0	5.82	17

* Sephadex G-100 fraction no. 8 (0.01 mg/ml). This fraction contained no measurable HCG activity.

‡ Extracellular infectivity measured 6 days (CMV) and 48 h (HSV-1) after viral inoculation.

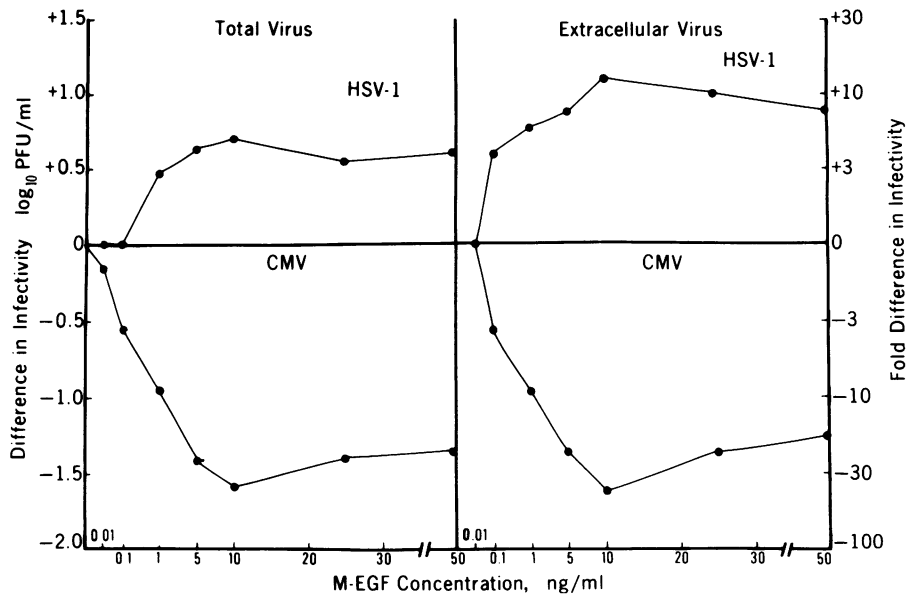


FIGURE 2 The effect of varying the concentration of m-EGF on the production of total and extracellular infectious CMV and HSV-1 in human fibroblasts. Viral MOI was 0.1 PFU/cell for both viruses. Infectivity titers were determined at 48 h postinoculation with HSV-1 and at 6 days with CMV. Effect of hormone on growth of the viruses was measured as $\log_{10}$ PFU/ml differences in infectivity generated in treated and untreated control cells. The amount of infectious virus in control cultures at the times of comparison were as follows: CMV, total 5.61 $\log_{10}$ PFU/ml, extracellular 5.22 $\log_{10}$ PFU/ml; HSV-1 total 5.51 $\log_{10}$ PFU/ml, extracellular 3.72 $\log_{10}$ PFU/ml. Each experiment was performed in duplicate and run concomitantly in the same batch of cells. Variability in duplicate runs were insignificant so that average values are shown. A second dose response experiment gave essentially the same results as depicted here.

of maximal virus production in control cultures (48 h after infection in this experiment). In 26 experiments, maximal enhancement of HSV-1 extracellular infectivity with m-EGF ranged from 0.88 to 1.42 (average = 1.02±0.23) logs. Pretreatment of cultures was required to elicit both the suppressive and enhancement phenomena. The data summarized in Fig. 4 indicated that the maximal effects required 24–36 h of pretreatment and neither response was seen when treatment was started with or after infection. In summary, the biologic effects of m-EGF on CMV and HSV-1 growth were virtually the same as those of crude HCG.

Adsorption experiments were next performed to conclusively prove that EGF was the material in crude HCG responsible for the alteration of the growth of CMV and HSV-1. After adsorption of crude HCG or active Sephadex fraction no. 8 with either specific hyperimmune sera to m-EGF or h-EGF, the suppression of CMV replication caused by these preparations (1.64 and 1.69 logs, respectively) was virtually eliminated. Likewise, the increase in HSV-1 replication caused by each of these preparations (0.99 and 1.17 logs, respectively) was likewise ablated. Neither normal rabbit serum nor specific antibody controls had an appreciable effect on the growth of either CMV or HSV-1 indicating the specificity of the adsorption reactions.

Having established that EGF was the active principal in crude HCG responsible for the alteration of productive CMV and HSV-1 infections, experiments were undertaken to better define its mode of action on the replication of CMV at the cellular level. Neutralization experiments indicated that m-EGF, like crude HCG, in concentrations (10 and 50 ng/ml) capable of causing maximal suppression of CMV growth, had no effect on extracellular virus. After 1 h of preinfection incubation, plaque production of CMV alone (112±6 PFU/0.05 ml) was similar to plaque production of CMV mixed with m-EGF (104±4 PFU/0.05 ml). In contrast, human serum with a CMV neutralizing antibody titer of 1:256 completely neutralized infectivity (0.0 PFU/0.05 ml). After penetration, crude HCG and m-EGF apparently have an effect on the cell such that CMV replication is blocked. Employing sequential immunofluorescent analysis for specific CMV antigen development over a 24-h period after virus inoculation, both compounds were observed to arrest viral antigen development in the early nuclear phase (Fig. 5*a*). Neither later nuclear nor specific cytoplasmic fluorescence patterns (Fig. 5*b*) developed as they did in un-

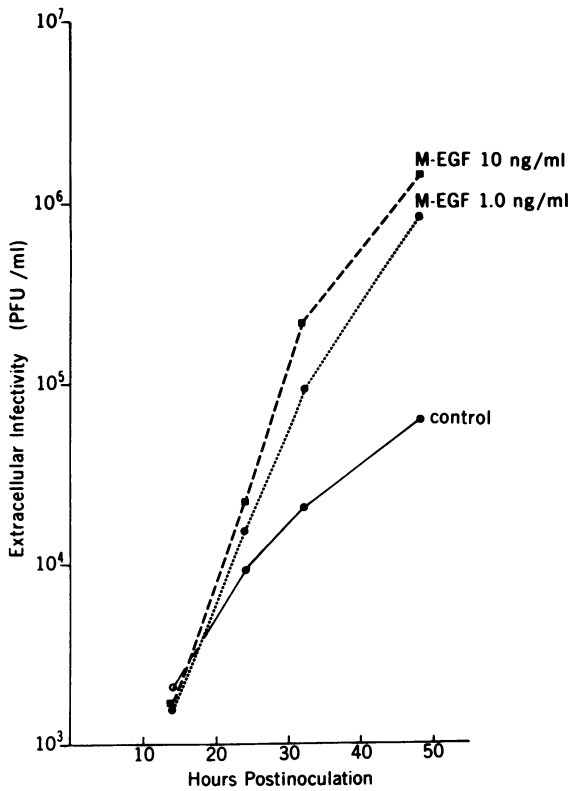


FIGURE 3 Effect of m-EGF on growth of HSV-1 in human fibroblast cells. MOI was 0.1 PFU/cell. Infectivity measurements are described in Methods. Control viral production as well as infectivity differences between control and treated wells were maximal 48 h after viral inoculation. Absolute titers (treated and control) at 72 and 96 h were equal to and less than 48 h, respectively.

treated infected cells 24 h postinfection. Instead, the nuclear fluorescence pattern after pretreatment with crude HCG or m-EGF remained both qualitatively and quantitatively the same (arrested at the 4-h stage, Fig. 5a) as that seen when the fibroblasts were pretreated with purified interferon, a material known to interfere with early events in viral replication. That the suppression of CMV growth by m-EGF was dependent upon cellular protein but not DNA synthesis is expressed in the data shown in Table IV. Simultaneous pretreatment of cells with cycloheximide and m-EGF abolished the latter's suppressive effect, whereas pretreatment with ara-C and m-EGF failed to do so. Pretreatment with cycloheximide or ara-C alone did not effect replication of infectious CMV.

DISCUSSION

To our knowledge, this is the first demonstration of the ability of a naturally occurring peptide hormone to alter productive viral infection in vitro or in vivo. Though purified m-EGF was used to delineate the effects on viral growth, the human counterpart contained in crude HCG preparations exerted the same biologic reactivities. This finding is not particularly surprising because the mouse and human hormones are almost identical, both chemically and antigenically, and are not apparently species specific with respect to other biologic activities in animal and cell culture systems (12). Clearly, their capacity to alter growth of CMV and HSV-1 also lacks species specificity, as evidenced here by the ability of antibody to m-EGF and h-EGF to ablate in a like manner the effects of h-EGF contained in crude HCG. The striking similarities between the cellular effects of the mouse and human hormones should greatly simplify current studies aimed at defining molecular events involved in the alteration of viral growth because purified m-EGF can presently be produced in far greater quantities than h-EGF.

The suppressive effect of EGF on the growth of CMV is similar in some respects to the effects of interferon on this and other viruses (15, 16). Neither compound apparently alters extracellular infectivity

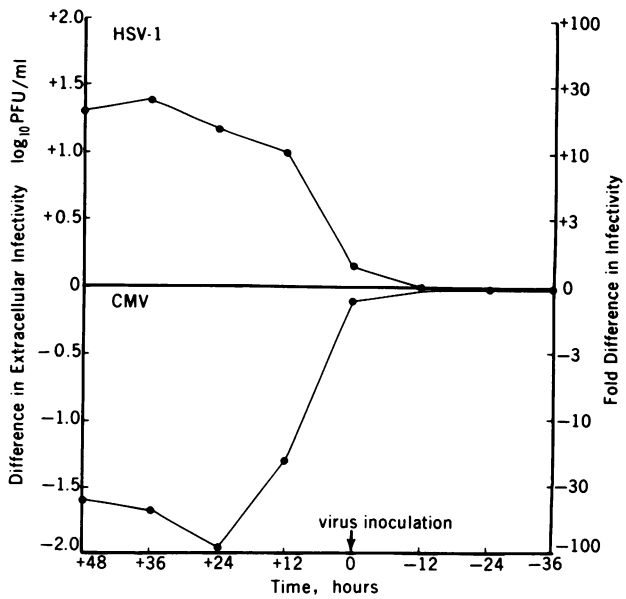
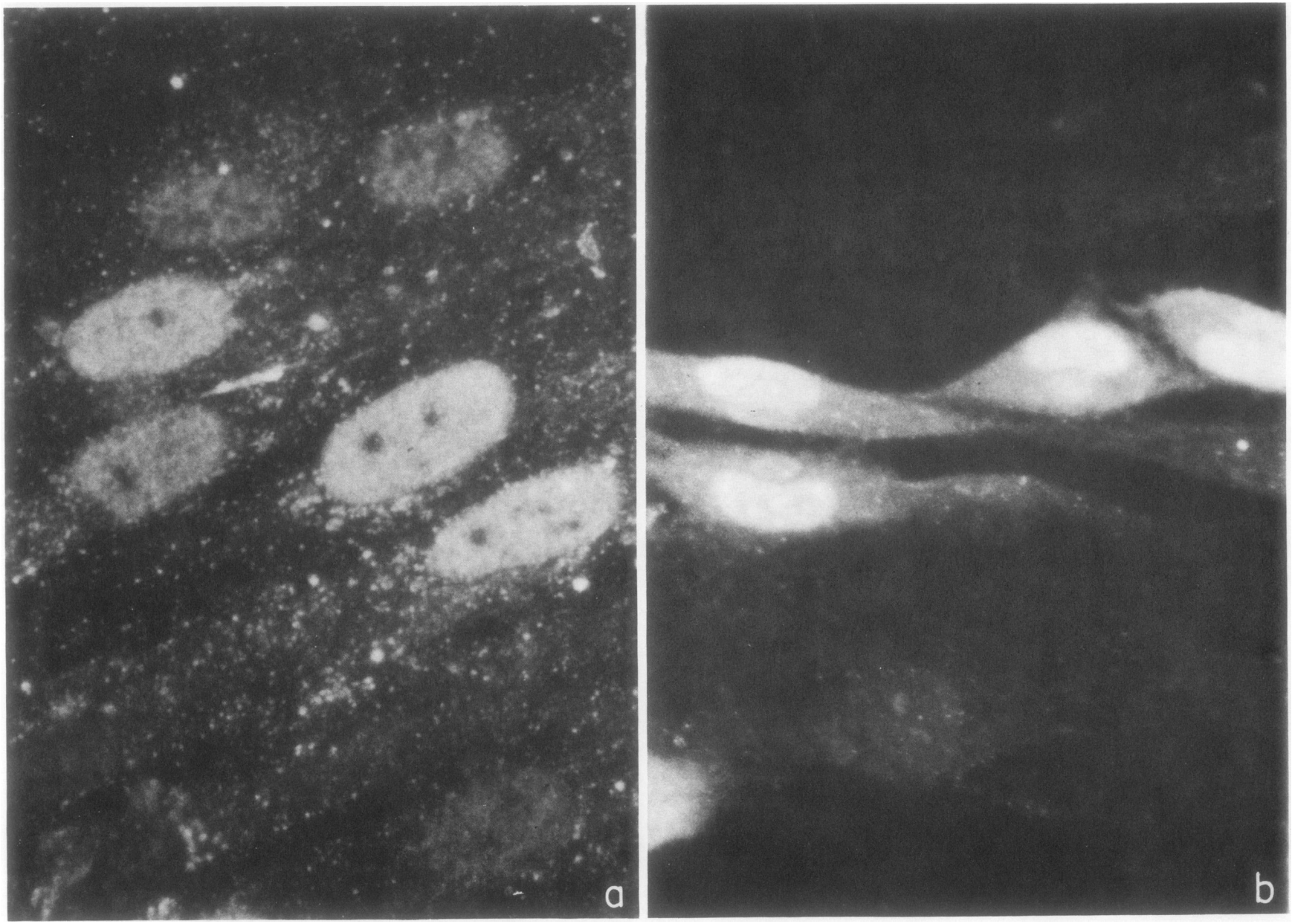


FIGURE 4 Comparative effects of varying treatment schedule with m-EGF (10 ng/ml) on the growth of extracellular infectious CMV and HSV-1 in human fibroblasts. Viral MOI was 0.1 PFU/cell with both agents. Infectivity titers were determined at 48 h postinoculation with HSV-1, and at 6 days with CMV. Effect of hormone on the growth of the viruses was measured as $\log_{10}$ PFU/ml differences in extracellular infectivity generated in treated and untreated control cells. The amount of extracellular infectivity in the control cultures at the times of comparison was 4.81 $\log_{10}$ PFU/ml for HSV-1 and 5.07 $\log_{10}$ PFU/ml for CMV. The experiment was performed in duplicate and run concomitantly in the same batch of cells. Variability in duplicate runs was insignificant so that only average values are shown.


a
b

or cellular absorption of virus. Both interfere with early intracellular events in viral replication. That is with CMV, early-nuclear, fluorescent antigens, which appear 1–4 h after infection, form in their presence, but later-nuclear and cytoplasmic antigens, as well as infectious virus, fail to do so. Moreover, suppression of CMV growth by interferon and EGF is dependent upon cellular protein but not DNA synthesis, and both require cell priming (pretreatment) to be effective. However, purified fibroblast interferon and poly(I):poly(C) suppress the growth of VSV, CMV, and HSV-1 in human fibroblasts, whereas EGF enhances the growth of HSV and, to a lesser extent, that of VSV. Thus, the CMV suppressive effect of EGF in crude HCG does not appear to be mediated by interferon or interferon induction.

The precise cellular mechanisms leading to suppression of CMV and enhancement of HSV-1 growth by EGF are presently unknown. The parallelism detected here between stimulation of cell growth and alteration in viral growth suggests that increased cellular metabolism may be a controlling factor. Indeed, with regard to suppression of CMV growth, DeMarchi and Kaplan have shown that replication of viral antigens and therefore infectious CMV are reduced when cellular DNA synthesis is stimulated, presumably in their work, by defective CMV virions (17). Conversely, inhibition of cellular DNA synthesis by 5-iodo-2′-deoxyuridine or caffeine treatment causes an increase in productive CMV infections, as shown by St. Jeor and Rapp (18) and Yamanishi et al. (19), perhaps due to the reduced formation of a normal cellular factor which suppresses CMV replication (20). Our findings lend credence to this concept. The increased cellular metabolism, especially protein synthesis, associated with the mitogenic effect of EGF could lead to an increase in the putative suppressing factor with consequent reduction of CMV growth in stimulated cells. However, enhancement of HSV-1 infection certainly cannot be ascribed to such a mechanism, suggesting that the effect of EGF on herpesvirus growth, whether connected to increased cellular metabolism or not, is likely mediated by complex independent mechanisms. These findings all focus on the need to better understand the role of the cell in controlling herpesvirus infections and how it might be manipulated for clinical advantage.

The discovery of EGF in crude HCG preparations in biologically active quantities and its effects on viral and cellular growth in vitro suggests a number of theoretical concerns in the clinical use of the crude preparations. Crude HCG is widely employed to treat various human conditions but the possible biological side effects of its contaminants remain largely unknown. Other contaminants, other than EGF according to our unpublished observations, have already been shown to alter lymphocyte and complement reactivities in vitro (7, 8) suggesting a possible immunosuppressive effect in vivo. This property, coupled with the ability of EGF to enhance the growth of HSV-1, could lead to a number of adverse side effects including, importantly, increased production of infectious virus and disease in various organs and increased oncogenic potential in the genital tract. In the case of carcinogenesis, the mitogenic effect of h-EGF, found in crude HCG could be deleterious, especially if hormonal therapy is to be used repeatedly or over long periods. Though it is a quantum leap to attempt translation of in vitro studies to the intact animal, these findings do emphasize the need to critically evaluate the entire spectrum of the biologic activities of crude hormonal preparations in cells and animals before their widespread use in man, and to constantly upgrade purification of commercial products. Even with purified

TABLE IV

Effect of Ara-C and Cycloheximide* on the Ability of m-EGF* to Suppress Growth of CMV*

Pretreatment‡	Change in CMV infectivity	
	PFU/ml (log_{10})	*Fold*
Control (5.60 log_{10} PFU/ml)§		
m-EGF	−1.67	−47.0
m-EGF + ara-C‖	−1.40	−25.0
ara-C‖	−0.20	−1.60
m-EGF + cycloheximide¶	+0.05	+1.12
Cycloheximide¶	+0.23	+1.50

* Concentrations: m-EGF = 10 ng/ml, ara-C = 20 μg/ml, cycloheximide = 20 μg/ml.

‡ Pretreatment 24 h, then all compounds removed before viral inoculation.

§ Infectivity level of untreated culture at time of comparison 6 days after viral inoculation.

‖ Compared to controls, fibroblasts treated with ara-C (20 μg/ml) had a 96.5% reduction of DNA synthesis as measured by cellular incorporation of [^{14}C]thymidine over 24 h.

¶ Compared to controls, fibroblasts treated with cycloheximide (20 μg/ml) had a 90.2% reduction of protein synthesis as measured by cellular incorporation of [^{3}H]leucine (leucine-free media) over 24 h.

FIGURE 5 (*a*) Fluorescence photomicrograph of nuclear antigen detected in untreated human fibroblast cells infected with human cytomegalovirus 4 h postinfection with the anti-IgG immunofluorescent technique. (*b*) Fluorescence photomicrograph of nuclear and cytoplasmic antigens detected in untreated human fibroblast cells infected with human cytomegalovirus 24 h postinfection with the anti-IgG immunofluorescent technique.

compounds employed exclusively for research purposes, deleterious side effects may be unavoidable. Certainly, the effect of any natural or artificial cell stimulant, including EGF, on growth of other viruses, productive and latent, should be more thoroughly investigated before utilizing these substances as cell growth stimulants for diagnostic or research tissue-culture purposes.

These studies were initially provoked by epidemiologic observations suggesting suppression of productive cervical CMV infection in early human pregnancy. The fact that EGF is able to suppress productive CMV infection in vitro and is found in lyophilized, first-trimester pregnancy urine neither supports nor refutes this hypothesis. In fact, these in vitro experiments may have little or nothing to do with the in vivo situation. Because EGF has been found in male and nonpregnant female urine (21), initial clarification of any physiologic function specific to pregnancy awaits quantitative determinations of its concentration at various stages of human gestation. It is known that the amount of EGF synthesized in mice is influenced by another hormone, namely testosterone (22). Therefore, the possible influence of human pregnancy hormones is currently under study in cell culture systems in hopes of better defining interactions between these substances and EGF on growth of herpesviruses. Aside from its effects on viral growth, the physiologic role of EGF in man, most particularly as regards cell growth, differentiation, and reproduction, remain fascinating topics for future research.

ACKNOWLEDGMENTS

The research for this report was supported by grants from the National Institute of Child Health and Human Development (program project HD10699, HD00700, and training grant HD00413).

REFERENCES

1. Stagno, S., D. W. Reynolds, A. Tsiantos, D. A. Fuccillo, R. Smith, M. Tiller, and C. A. Alford, Jr. 1975. Cervical cytomegalovirus excretion in pregnant and nonpregnant women: suppression in early gestation. *J. Infect. Dis.* **131:** 522–527.
2. Numazaki, Y., N. Yano, T. Morizuka, S. Takai, and N. Ishida. 1970. Primary infection with human cytomegalovirus: virus isolation from healthy infants and pregnant women. *Am. J. Epidemiol.* **91:** 410–417.
3. Montgomery, R., L. Youngblood, and D. N. Medearis, Jr. 1972. Recovery of cytomegalovirus from the cervix in pregnancy. *Pediatrics.* **49:** 524–531.
4. Jordan, M. C., W. E. Rousseau, G. R. Noble, J. A. Stewart, and T. D. Y. Chin. 1973. Association of cervical cytomegaloviruses with venereal disease. *N. Engl. J. Med.* **288:** 932–934.
5. Katzman, P. A., M. Godfrid, C. K. Cain, and E. A. Doisy. 1943. The preparation of chorionic gonadotropin by chromatographic adsorption. *J. Biol. Chem.* **148:** 501–507.
6. Tulchinsky, D., and C. J. Hobel. 1973. Plasma human chorionic gonadotropin, estrone, estradiol, estriol, progesterone, and 17-hydroxy-progesterone in human pregnancy. *Am. J. Obstet. Gynecol.* **117:** 884–893.
7. Caldwell, J. L., D. P. Stites, and H. H. Fudenberg. 1975. Human chorionic gonadotropin: effects of crude and purified preparations on lymphocyte responses to phytohemagglutin and allogenic stimulation. *J. Immunol.* **115:** 1249–1253.
8. Loke, Y. W., and M. B. Pepys. 1975. Effects of human chorionic gonadotropin preparations on complement in vitro. *Am. J. Obstet. Gynecol.* **121:** 37–40.
9. O'Dell, W. D., and G. T. Ross. 1971. Correlation of bioassay and immunoassay potencies for FSH, LH, TSH, and HcG. *In* Principles of Competitive Protein Binding Assays. W. D. Odell and W. H. Daughaday, editors. J. B. Lippincott Co., Philadelphia, Pa. 401–407.
10. Savage, C. R., Jr., and S. Cohen. 1972. Epidermal growth factor and a new derivative. Rapid isolation procedures and biological and chemical characterization. *J. Biol. Chem.* **247:** 7609–7611.
11. Carpenter, G., K. J. Lembach, M. M. Morrison, and S. Cohen. 1975. Characterization of the binding of ^{125}I-labeled epidermal growth factor to human fibroblasts. *J. Biol. Chem.* **250:** 4297–4304.
12. Cohen, S., and G. Carpenter. 1975. Human epidermal growth factor: isolation and chemical and biological properties. *Proc. Natl. Acad. Sci. U. S. A.* **72:** 1317–1321.
13. Wentworth, B. B., and L. French. 1970. Plaque assay of cytomegalovirus strains of human origins. *Proc. Soc. Exp. Biol. Med.* **135:** 253–258.
14. Geder, L. 1976. Evidence for early nuclear antigens in cytomegalovirus-infected cells. *J. Gen. Virol.* **32:** 315–319.
15. Finter, N. B., editor. 1973. *In* Interferons and interferon inducers. North-Holland Co., Amsterdam and London.
16. Knox, G. E., D. W. Reynolds, and C. A. Alford. 1977. Sensitivity of cytomegalovirus to human interferon. *Clin. Res.* **25:** 78A. (Abstr.)
17. DeMarchi, J. M., and A. S. Kaplan. 1977. The role of the physiological state of cells and the "quality" of virus in CMV induced cellular DNA synthesis. Third International Symposium on Oncogenesis and Herpesviruses, Program and Abstracts. 85.
18. St. Jeor, S., and F. Rapp. 1973. Cytomegalovirus replication in cells pretreated with 5-iodo-2'-deoxyuridine. *J. Virol.* **11:** 986–990.
19. Yamanishi, K., M. Fogel, and F. Rapp. 1977. Effect of caffeine on cytomegalovirus replication. Abstracts of the Annual Meeting of the American Society for Microbiology. S384.
20. Glaser, R., J. Zimmerman, S. St. Joer, and F. Rapp. 1975. Demonstration of a cellular inhibitor of Epstein-Barr and cytomegalovirus synthesis. *Virology.* **64:** 289–292.
21. Starkey, R. H., S. Cohen, and D. N. Orth. 1975. Epidermal growth factor: Identification of a new hormone in human urine. *Science (Wash. D. C.).* **189:** 800–802.
22. Cohen, S., and J. M. Taylor. 1972. Part I. Epidermal growth factor: chemical and biological characterization. *In* Epidermal Wound Healing. H. I. Maibach and D. T. Rovee, editors. Year Book Medical Publishers, Chicago, Ill. 203–218.